0273-2289/98/70–72—0929$9.75

Accumulation of Biodegradable Copolyesters of 3-Hydroxy-Butyrate and 3-Hydroxyvalerate in *Alcaligenes eutrophus*

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ABSTRACT

Biodegradable copolyesters of 3-hydroxybutyrate-co-3-hydroxyvalerate (3HB-3HV) were produced by *Alcaligenes eutrophus* in a two-staged process, namely growth stage and nitrogen-deficient polyester-accumulation stage. When C_5 was used as the sole carbon source, the copolyester contained 43 mol % of 3HV. A range of copolyesters with 0–43 mol % of 3HV could be produced by using a medium containing different concentration ratios of butyric acid C_4 and C_5. T_m of PHB homopolymer was 177.6°C and that of copolyester with highest 3HV mol fraction of 43% was 99.0°C. C_5 concentration in the medium could be an effective means to control the polymeric composition and mechanical properties of the copolyesters accumulated in *A. eutrophus*.

Index Entries: *Alcaligenes eutrophus* H16; biodegradable plastics; physical properties; poly (3-hydroxybutyrate-co-3-hydroxyvalerate).

INTRODUCTION

Over the past two decades, plastic usage and plastic-waste generation have been on a drastically increasing trend, and are forecast to increase at a rate of 15% a year over the next decade *(1–4)*. Commonly used plastics, including high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC), are synthetic polymeric hyrocarbons derived from petroleum, and are not easily decomposed by microorganisms *(5–8)*. Plastic wastes are therefore considered to be among

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the most environmentally harmful wastes and have led to two environmental concerns.

Microbial polyesters and copolyesters, namely poly-hydroxyalkanoates (PHAs), have been recognized as a potential environment-friendly substitute for traditional plastics *(9–12)*. A number of bacteria, including *Alcaligenes*, *Pseudomonas*, recombinant *Escherichia coli*, and a few filamentous genera, accumulate these polyesters or copolyesters as an intracellular carbon reserve when unfavorable environmental conditions are encountered *(13–16)*. The extracted and processed polymeric substances have properties that are comparable to commonly used PE and PP, namely thermoplastic processability and 100% resistance to water. In addition, PHAs are completely biodegradable in natural environments *(17)*.

In recent years, much effort has been spent in optimizing the PHA production process and reducing costs *(18–20)*. Chua et al. *(21,22)* demonstrated a novel technique to produce PHA copolyesters as a byproduct from conventional activated sludge wastewater treatment process. However, an important aspect that has to be addressed before widespread applications of PHAs in packaging and disposable products are possible is the improvement of the physical and mechanical properties. *Alcaligenes eutrophus* produces PHB homopolyester and 3HB-3HV copolyester from various carbon sources, including glucose, fructose, and organic acids *(23)*. When even-numbered carbon compounds, such as glucose, acetic, butyric, and caproic acids, were used as the carbon source, only PHB was produced. When odd-numbered carbon compounds, such as propionic and valeric acids and propanol, were used as the carbon source, different 3HB-3HV copolyesters could be produced. PHB homopolymer was a relatively stiff and brittle material because of high crystallinity, and the melting point was 177°C. On the other hand, the physical properties of 3HB-3HV copolyesters, including melting point, mechanical strength, and biodegradability, were widely varied and dependent on the mole fractions of monomeric units in the copolyester *(24–28)*. Unfortunately, information on the control of HV fraction in the copolyester production that improve mechanical properties and decrease melting point are seriously lacking.

In this paper, butyric and valeric acids were used as carbon sources to produce PHA in a two-stage cultivation of *A. eutrophus*. The relationship between butyric acid (C_4) to valeric acid (C_5) ratio and the 3HV fraction in and the physical property of the copolyester was investigated.

MATERIALS AND METHODS

Bacterial Cell Line and Growth Medium

A. eutrophus H16 (American Type Culture Collection ATCC 17966) was used to generate copolyesters. The first stage of the two-stage cultivation was a cell-growth stage using a nutrient-rich medium, consisting of

10 g/L yeast extract, 10 g/L polypeptone, 5 g/L meat extract, and 5 g/L ammonium sulphate, in a 500-mL shaker flask at 30°C and shaken at 160 rpm for 24 h.

The second stage was a nutrient-deficient copolyester accumulation stage. Cells from the first stage were harvested by centrifugation and washed with buffer solution to remove any residual nitrogenous matters. About 7.5 g of the harvested and washed cells was transferred into a jar fermentor filled with a nitrogen-free medium. The medium contained C_4 and/or C_5 as the carbon sources, supplementary trace minerals and a growth factor with formulations previously described by Chua et al. *(22)*. In separate batch cultures, the C_4 to C_5 weight ratios in the medium were respectively adjusted to 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. The initial total concentration of fatty acids was adjusted to 1 g/L to avoid possible growth inhibition as reported by Kim et al. *(29)*. The fermentor was operated in a fed-batch mode by adding fatty acids into the fermentor once every 16 h to maintain the total acid concentration at a level equal to or lower than 1 g/L.

Fermentation Conditions

The automatic jar fermentation (Bioengineering Model ALF, Ruti/Switzerland) was of a 3-L working volume and operated at 300 rpm and 30°C for 48 h. The pH of the culture medium was automatically maintained at 7.0 by the addition of a sterilized 2 *M* NaOH solution or 2 *M* H_2SO_4 solutions.

Sampling and Analytical Techniques

The culture broth was periodically sampled and analyzed for residual carbon concentration and dry cell mass. Residual carbon concentration was measured as total organic carbon (TOC) using an Astro 2001 System 2 automatic TOC analyzer. The TOC analytical technique and dry cell-mass measurement procedure were in accordance with the standard methods *(30)*.

The copolyesters accumulated in the cells were extracted by using a Soxhlet extractor with 200 mL of chloroform at 70°C for 24 h. The extracted copolyesters were purified by precipitation with 200 mL of methanol and weighed with an analytical balance. Thermal property of the extracted copolyesters was analyzed by a digital melting-point apparatus (electrothermal digital melting point apparatus Model IA9100). The extraction and analytical procedures were according to that described by Ho *(10)*.

The accumulated copolyesters were also extracted by a different procedure for composition analysis. Culture broth (10 mL) was sampled and centrifuged at 3000 rpm for 15 min. The settled cells were washed with buffer solution and centrifuged again. The settled cells were then resuspended in a mixture containing 2 mL of methanol with 3% of concentrated

H_2SO_4 and 2 mL of chloroform with 2 mg/mL of benzoic acid as the internal standard. The sample was placed in a closed test tube and heated at 100°C for 3 h to convert the 3HB and 3HV constituents into their methylesters. The composition of these extracts was then analyzed by gas chromatographic techniques using a Varian model 3700 gas chromatograph equipped with a Carbowax 20 *M* column (1 meter) and a Shimazu C-R5A Chromatopac flame-ionization detector. The analytical procedure was according to that described by Ho *(10)*.

RESULTS AND DISCUSSION

Cell Growth and Polymer Production at Various Fatty Acid Ratios

The initially inoculated cell mass in fermentor was approx 7.5 g and the final cell mass ranged from 7.8 to 9.0 g in different batches of cultures with varied fatty acid ratios. The overall cell mass in the nutrient-deficient stage remained almost unchanged throughout the 48-h operation. An increase in C_5 concentration in the medium from 0 to 100 weight % resulted in a decline in both the specific copolyester yield, $Y_{p/x}$, from 0.41 to 0.04 g-copolyester/g-cell, and the copolyester production yield, $Y_{p/s}$, from 0.41 to 0.06 g-copolyester/g-TOC consumed (Table 1). These results indicated inhibitory effect of C_5 on the production of copolyesters. The highest $Y_{p/s}$ of 0.41 g/g achieved was within the theoretical maximum value of 0.65 g/g for *A. eutrophus (16)*. However, under the subinhibitory concentration of total fatty acid (less than 1.0 g/L) in the medium, the observed decline of $Y_{p/s}$ with increasing C_5 mol ratio was in contrast to that reported by Ishihara et al. *(31)* that $Y_{p/s}$ remained largely unchanged regardless of C_5 ratio in the medium.

Effect of Fatty Acid Ratio on 3HV Fraction in Copolyesters

Table 1 shows the effect of C_5 concentration in the medium on the 3HV-mole fraction of PHA. When C_4 was used as the sole carbon source, only PHB homopolyester (0 % 3HV) was produced without any 3HB-3HV copolyesters. On the other hand, a highest 3HV-mole fraction of 43 mol % in the copolyester accumulated was obtained when C_5 was used as the sole carbon source. The mole fraction of 3HV in the accumulated copolyesters increased with the increasing C_5 concentration in the medium (Table 1). The relationship between 3HV-mole fraction in the 3HB-3HV copolyesters and the C_5 concentration in the medium was linear. If the 3HV-mole fraction was represented by Y_1 and the C_5 concentration was represented by X_1, then the correlation could be expressed in *equation (1)* as follows.

$$Y_1 = 0.464\ X_1 + 0.400,\ r^2 = 0.956 \quad (1)$$

These results were in agreement with that observed in other works *(31,32)*. The changes of 3HB and 3HV mole fractions in the copolyesters

Table 1
Copolyester Accumulation Under Different Fatty Acid Ratios

Carbon Conc. (g/L) C_4	Carbon Conc. (g/L) C_5	C_4 to C_5 Ratio	$Y_{p/x}$[1] (g/g)	$Y_{p/s}$[2] (g/g)	HV Fraction (mol %)	T_m (°C)
3.0	0.0	100:0	0.41	0.41	0	177.6
2.4	0.6	80:20	0.36	0.18	11	149.0
1.8	1.2	60:40	0.32	0.15	27	136.0
1.2	1.8	40:60	0.26	0.12	33	129.5
0.6	2.4	20:80	0.06	0.10	41	116.0
0.0	3.0	0:100	0.04	0.06	43	99.0

accumulated were attributed to the beta-oxidation metabolic pathway for fatty acids. When C_5 was used as the carbon source, acetyl-CoA and propionyl-CoA were produced from 3-keto-valeryl-CoA and both 3HB and 3HV monomeric units were produced. On the other hand, when C_4 was used as the sole carbon, propionyl-CoA was not produced, and only 3HB monomeric units were produced. These results also indicated that C_5 concentration in the medium could be an effective means to control the 3HV-mole fraction in the copolyesters accumulated in *A. eutrophus*.

Melting Temperature of Various Copolyesters

The melting temperature, T_m, of the copolyesters accumulated by *A. eutrophus* with different fatty acid ratios in the medium ranged from 99.0 to 177.6°C (Table 1). The highest value of T_m (177.6°C) was that of PHB, which was produced when C_4 was used as the sole carbon source in the medium. This product was of relatively high crystallinity, resulting in high stiffness and brittleness (Fig. 1). On the other hand, the lowest value of T_m was that of copolyester with the highest 3HV-mole fraction of 43 %, which was produced when C_5 was used as the sole carbon source in the medium. Therefore, the reduced crystallinity with the increasing of 3HV monomeric units in the copolyesters improved such mechanical properties as tensile, shear, compressive, and flexural strengths (Fig. 2). Results in Table 1 show that the T_m decreased with the increasing of 3HV monomeric units in the copolyesters. The relationship between T_m and 3HV-mol % was linear. If T_m was represented by Y_2 and the 3HV-mol % was represented by X_2, then the correlation could be expressed in equation (2) as follows.

$$Y_2 = 1.547\ X_2 + 174.470,\ r^2 = 0.938 \quad (2)$$

These results also indicated that C_5 concentration in the medium could be an effective means to control the mechanical properties of the copolyesters accumulated in *A. eutrophus*.

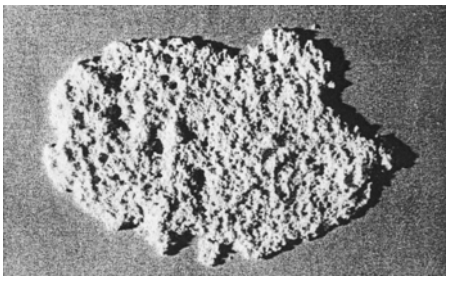

Fig. 1. Sample of PHB showing high crystallinity resulting in high stiffness and brittleness.

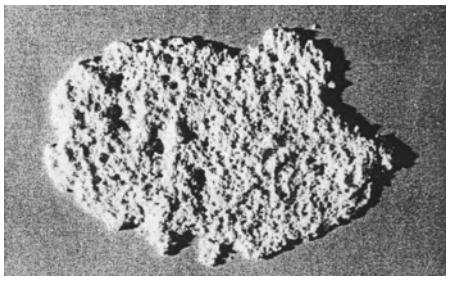

Fig. 2. Sample of 3HB-3HV copolyesters 43% of 3HV mole fraction showing reduced crystallinity resulting in improved mechanical properties.

CONCLUSION

When C_5 was used as the sole carbon source, the copolyester contained 43 mol % of 3HV. A range of copolyesters with 0 to 43 mol % of 3HV could be produced by using a medium containing different concentration ratios of butyric acid C_4 and C_5. T_m of PHB homopolymer was 177.6°C and that of copolyester with highest 3HV mol fraction of 43% was 99.0°C. C_5 concentration in the medium could be an effective means to control the polymeric composition and mechanical properties of the copolyesters accumulated in *A. eutrophus*.

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